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Predicting *In Vivo* Efficacy of Therapeutic Bacteriophages Used To Treat Pulmonary Infections

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The potential of bacteriophage therapy to treat infections caused by antibiotic-resistant bacteria has now been well established using various animal models. While numerous newly isolated bacteriophages have been claimed to be potential therapeutic candidates on the basis of *in vitro* observations, the parameters used to guide their choice among billions of available bacteriophages are still not clearly defined. We made use of a mouse lung infection model and a bioluminescent strain of *Pseudomonas aeruginosa* to compare the activities *in vitro* and *in vivo* of a set of nine different bacteriophages (PAK_P1, PAK_P2, PAK_P3, PAK_P4, PAK_P5, CHA_P1, LBL3, LUZ19, and PhiKZ). For seven bacteriophages, a good correlation was found between *in vitro* and *in vivo* activity. While the remaining two bacteriophages were active *in vitro*, they were not sufficiently active *in vivo* under similar conditions to rescue infected animals. Based on the bioluminescence recorded at 2 and 8 h postinfection, we also define for the first time a reliable index to predict treatment efficacy. Our results showed that the bacteriophages isolated directly on the targeted host were the most efficient *in vivo*, supporting a personalized approach favoring an optimal treatment.

Bacteriophages are viruses that infect bacteria. They are widespread in the environment, and bacteriophage therapy—the use of bacteriophages to treat bacterial infections—was first proposed almost 100 years ago (1). With the increasing frequency of antibiotic-resistant strains of pathogenic bacteria, there has been renewed interest in bacteriophage therapy as a promising alternative (2). The state of the art and the pros and cons of bacteriophage therapy have been extensively reviewed (3–6), and the data available for the current use of this approach to treat human patients in Poland and Georgia are encouraging (7, 8).

In this context, more efforts than ever before are being made to identify bacteriophages that infect various human pathogens, and these bacteriophages are often proposed as potential candidates for bacteriophage therapy as soon as their lytic nature has been established *in vitro* (9, 10). However, additional information should be obtained before considering the use of bacteriophages in human treatments, but unfortunately, to date, there is still no standardized method for evaluating the therapeutic potential of bacteriophages. For instance, in a complex environment, such as the human body, various factors (immune cells, enzymes, peptides) may interfere with or even abolish bacteriophage activity, potentially rendering bacteriophages poor therapeutic candidates (11). The nature and presence of these factors also depend on the infectious site that is targeted, as, for example, immune defenses may vary between organs (12).

Host-virus interaction studies have suggested that the rate of bacterial killing, the dose, and the presence of bacteriophage-encoded enzymes are determinants involved in treatment efficacy (13–17). Such parameters would be best evaluated in a single specific animal model with various bacteriophages. Unfortunately, to date, most studies assessing the efficacy of bacteriophages in animal models, including mice, sheep, cattle, pigs, and poultry (18–23), have rarely considered more than one bacteriophage at a time. Exceptions include a comparison of the replication pattern of two virulent bacteriophages in the gut (24) and, more recently, an evaluation of seven bacteriophages from two different classes in a model assessing the *in vivo* dynamics of bacteriophage replication (14). The few other studies conducted with several bacteriophages at a time focused on the use of cocktails of bacteriophages rather than comparisons of individual bacteriophages (25–27).

In this study, we compared nine bacteriophages infecting the same host, *Pseudomonas aeruginosa*, a major pathogen causing respiratory infections in immunodeficient individuals, including 80% of cystic fibrosis patients, for whom this bacterium constitutes the main cause of morbidity (28). These nine bacteriophages, including six closely related bacteriophages and three bacteriophages from other, different genera, were compared to PAK_P1, our reference bacteriophage previously reported to be effective in treating acute lung infection in a mouse model (29). The *in vitro* efficacy of each of these bacteriophages was compared to its *in vivo* efficacy in our lung infection model, in which bioluminescence was used for the real-time monitoring of infection, evidencing a good correlation between *in vitro* results and *in vivo* efficacy for seven bacteriophages.

MATERIALS AND METHODS

Bacteriophages and bacterial strains used in this study. The bacterial strains used in this work included the *P. aeruginosa* PAK strain, its bioluminescent version (PAK-lumi) (30), and the CHA strain (31).

The *Myoviridae* PAK_P1 (accession no. KC862297) (29), PAK_P2 (accession no. KC862298), PAK_P3 (accession no. KC862299) (32), PAK_P4 (accession no. KC862300), and PAK_P5 (accession no. KC862301) bacteriophages (a group referred to as the PAK_Px bacteriophages) were isolated from wastewater samples from the Paris, France, area with the PAK strain as a host. The member of the *Podoviridae* LUZ19 (44 kb; GenBank accession no. NC_010326.1) (33) and two members of the *Myo-*

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TABLE 1 Main characteristics of bacteriophages used in this study

Family	Genus	Name	Size (kb)	Host	EOP[a]	Accession no.
Myoviridae	Unclassified	PAK_P1	93	PAK	1	KC862297
	Unclassified	PAK_P2	91	PAK	1	KC862298
	Unclassified	PAK_P4	93	PAK	1	KC862300
	Unclassified	PAK_P3	88	PAK	1	KC862299
	Unclassified	PAK_P5	85	PAK	1	KC862301
	Unclassified	CHA_P1	88	CHA	0.5	KC862295
	Pbunalikevirus	LBL3	64	Aa245	0.8	NC_011165.1
	Phikzlikevirus	PhiKZ	280	PAO1	1.2	NC_004629.1
Podoviridae	*Phikmvlikevirus*	LUZ19	44	PAO1	0.2	NC_010326.1

[a] EOP values obtained on the PAK-lumi strain.

viridae, PhiKZ (280 kb; GenBank accession no. NC_004629.1) (34) and LBL3 (64 kb; GenBank accession no. NC_011165.1) (35), were amplified on strains PAO1 (LUZ19 and PhiKZ) and Aa245 (LBL3). The CHA_P1 bacteriophage (accession no. KC862295) was isolated from wastewater using the CHA strain (31). Briefly, for each bacteriophage, a 1-liter culture of the host (optical density [OD] at 600 nm in LB, 0.1) was infected with the given bacteriophage at a multiplicity of infection (MOI) of 0.001 and incubated at 37°C with shaking. Once lysis had occurred and the OD at 600 nm returned to a value below 0.1, the lysate was filter sterilized with two in-line filters (pore sizes, 0.8 to 0.45 and 0.2 to 0.1 μm; Sartopore 300; Sartorius) and concentrated with an ultrafiltration cassette (Vivaflow 200; Sartorius). The concentrates were then ultracentrifuged twice on cesium chloride gradients (36). The resulting solutions were then dialyzed against Tris buffer (10 mM Tris, 150 mM NaCl, pH 7.5), mixed with 0.03% gelatin, and stored at 4°C. CHA_P1 adaptation to the PAK strain was carried out as previously described (32). A detailed analysis of the PAK-Px genomes will be published elsewhere (M. Henry, L. M. Bobay, A. Chevallereau, E. Saussereau, P. J. Ceyssens, and L. Debarbieux, unpublished data).

***In vitro* tests.** Efficiency of plating (EOP) was determined on the same day on both the PAK-lumi strain and the original hosts for each bacteriophage using the standard plaque assay method. The EOP was calculated as the ratio of the number of plaques formed by each bacteriophage on the PAK-lumi strain to the number of plaques formed on its host. Lysis kinetics for each bacteriophage in liquid LB medium were performed using an MOI of 0.001, which represents the condition for which the clearest distinction between the different bacteriophages could be observed, and were determined using a 96-well plate reader (37).

Ethics statement. Eight-week-old BALB/c male mice (Janvier) were housed in animal facilities in accordance with French and European regulations on the care and protection of laboratory animals. Protocols were approved by the veterinary staff of the Institut Pasteur animal facility (approval number 10.565) and the National Ethics Committee (approval number 2012-0018).

***In vivo* tests.** Mice were infected by intranasal instillation of 1×10^7 CFU of PAK-lumi in 50 μl of PBS, following anesthesia by intramuscular injection of a mixture of ketamine and xylazine. Two hours later, the animals were treated by the same route with 30 μl of phosphate-buffered saline (PBS) or bacteriophage solution, following anesthesia by isoflurane inhalation delivered by an IVIS 100 imaging system (PerkinElmer). The titration of bacteriophage stocks was performed on the day before each experiment, and the exact bacteriophage dose given was also checked, to ensure that the MOI used was as accurate as possible. The survival and weight of the animals as well as the aspect of their fur and their motility were monitored daily for 13 days after infection. In accordance with the ethics policy relating to this protocol, the animals were euthanized when they were moribund or after they had lost more than 25% of their initial body weight.

Luminescence measurements. Luminescence was recorded as previously described by counting photons within a constant defined area corresponding to the surface of the chest and encompassing the whole lung region (29). All data were normalized by subtracting the background level obtained from a noninfected control animal included in each recording. Luminescence was measured for all animals at 2, 4, 6, and 8 h postinfection and then once every 2 days over a 13-day period. The ratio of the luminescence measurement at 8 h after infection to that at 2 h after infection was calculated as follows: the mean radiance value obtained for the noninfected animal was subtracted from the mean radiance value obtained for each animal of each group. The median value was then determined for each group for the 2- and 8-h-postinfection time points and used to calculate the ratio (luminescence at 8 h/luminescence at 2 h).

RESULTS

Isolation of PAK_Px bacteriophages. Bacteriophages infecting the PAK strain of *P. aeruginosa* were isolated from five environmental sources on the same day. Plaque morphology was used to select 16 candidates, from which we isolated genomic DNA. Restriction digestion of the DNA yielded five different restriction patterns. One representative of each pattern was chosen for further characterization and named PAK_P1 to PAK_P5 (here referred to as the PAK_Px bacteriophages). Only PAK_P1 and PAK_P2 originated from the same environmental source. The CHA_P1 bacteriophage was also later isolated from an environmental source, using as the host *P. aeruginosa* strain CHA, a clinical strain isolated from a cystic fibrosis patient (31). The genome sequences obtained for these bacteriophages showed that they formed two distinct subgroups, the first comprising PAK_P1, PAK_P2, and PAK_P4 and the second comprising PAK_P3, PAK_P5, and CHA_P1 (Table 1). A detailed analysis of these genomic sequences will be published elsewhere.

EOP and lysis kinetics. We first selected eight bacteriophages and evaluated their ability to form plaques on our model strain PAK-lumi of *P. aeruginosa* by determining their EOP on this strain. EOP was set to 1 for the PAK_Px bacteriophages and was found to be 0.8 for LBL3 and 0.2 for LUZ19. PhiKZ had an EOP of 1.2, as more plaques were observed on the PAK-lumi strain than on the strain used for its propagation, PAO1.

We then determined the lysis kinetics of these eight different bacteriophages on the PAK-lumi strain at the same MOI in liquid medium (Fig. 1A). The OD decreased after approximately 70 min of incubation for all the PAK_Px bacteriophages. However, we were able to differentiate two clusters within this group, the first consisting of PAK_P1, PAK_P2, and PAK_P4, for which the OD value reached 0.04 before being affected by bacterial lysis, and the second consisting of PAK_P3 and PAK_P5, for which the OD value reached 0.055 (Fig. 1A, inset). The time lag to a decrease in

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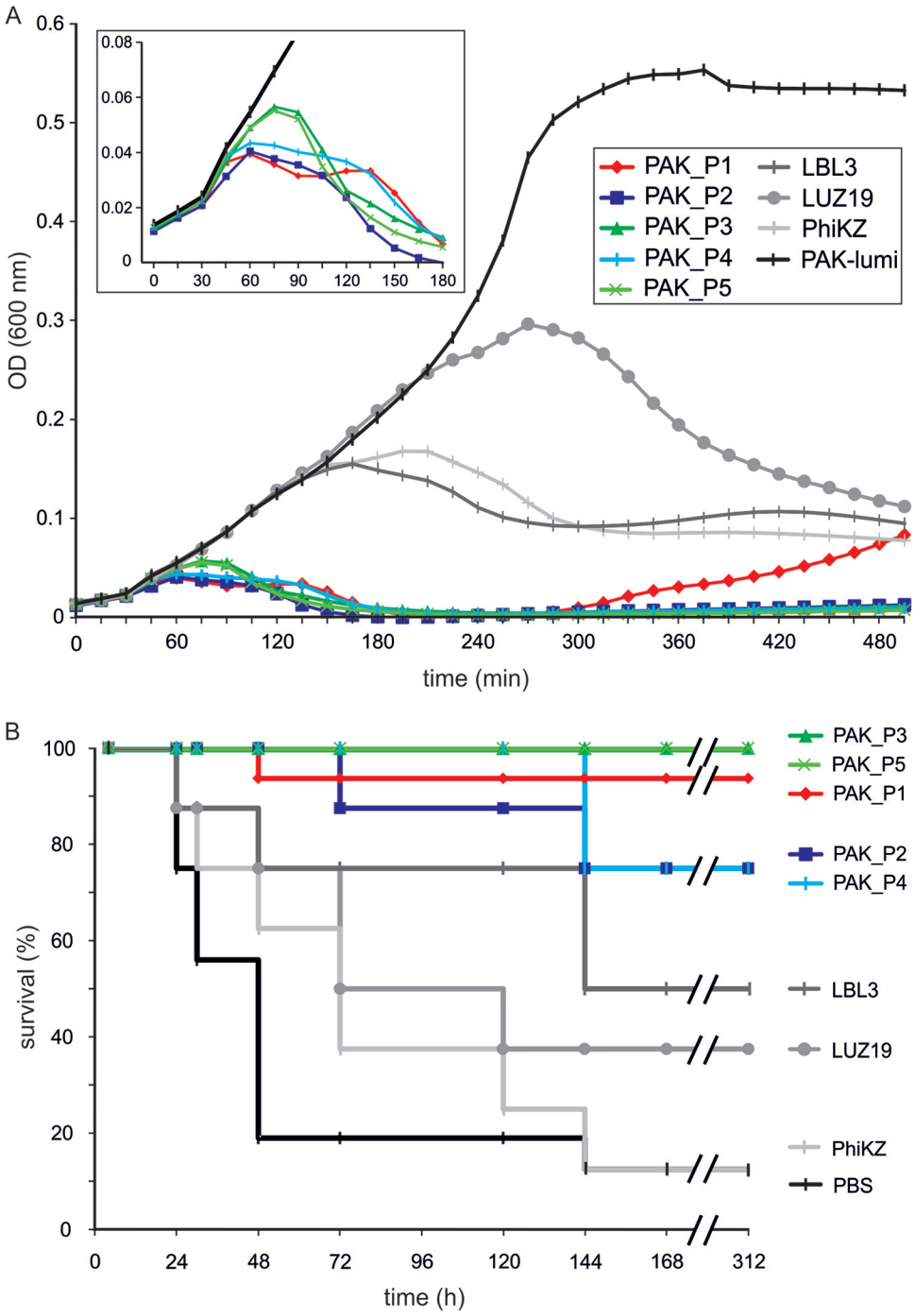

FIG 1 *In vitro* and *in vivo* activities of *P. aeruginosa* bacteriophages. (A) Individual *in vitro* lysis kinetics for the indicated bacteriophages at an MOI of 0.001 on the PAK-lumi strain (values are means of 4 replicates; only one set of data representative of 3 independent experiments is shown); (B) survival curves of the groups of animals infected with PAK-lumi and treated with the indicated bacteriophages at an MOI of 0.1. For each group of mice, the results shown are from at least two independent experiments (n = 8 to 16 animals).

the OD was the greatest for LUZ19 (about 270 min), suggesting that lysis occurred more slowly with this bacteriophage, whereas lysis occurred slightly more rapidly with PhiKZ and LBL3 (210 and 180 min, respectively).

Comparison of *in vivo* efficacies and short-term kinetics for curative bacteriophage treatments. We used the previously described bacteriophage PAK_P1 (29) as the reference for this study and refined the treatment dose for this phage to an MOI of 0.1 to obtain a survival rate of about 75%, making it possible to distinguish between bacteriophages with potentially higher or lower efficacies.

Using MOIs of between 0.05 and 0.2, we twice independently tested each of the bacteriophages (PAK_P2 to PAK_P5, LBL3, PhiKZ, and LUZ19) following the same procedure, with PAK_P1

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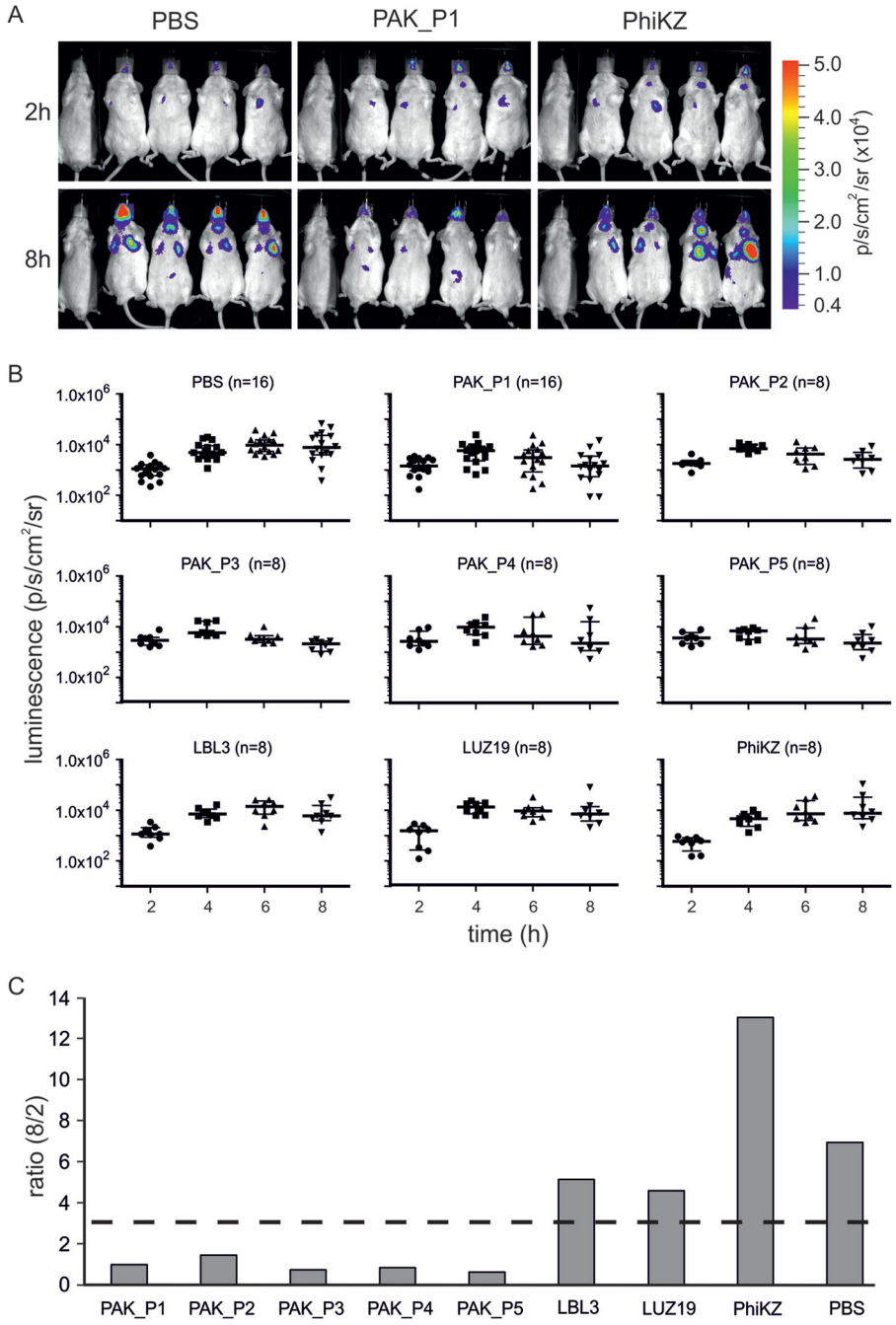

FIG 2 Short-term kinetics of bacteriophage treatments following infection with a lethal dose of the PAK-lumi strain (A) Representative images obtained at 2 and 8 h postinfection for groups treated with PBS, PAK_P1, or PhiKZ. The animal on the far left of each image is the noninfected mouse used for background light measurement. The scale was normalized to allow visual comparison. p/s/cm^2/sr, number of photons per second per square centimeter per steradian. (B) Average radiances for all the animals used in this study at each time point, with medians and interquartile range indicated. (C) Ratios of the median average radiance values at 8 h to those at 2 h for each group of mice.

and PBS included in each experiment as controls. The PAK_Px bacteriophages gave the highest survival rates, between 75 and 100% (Fig. 1B). We obtained survival rates of 50% for LBL3, 37% for LUZ19, and 15% for PhiKZ, a rate similar to that obtained with the PBS control.

By recording bioluminescence every 2 h for the first 8 h after infection, we were able to compare the short-term *in vivo* kinetics of treatments with the different bacteriophages by quantifying the luminescence emitted from the chest area of the animals (Fig. 2A and B). In all the groups, luminescence increased between 2 and 4 h after infection, confirming the active development of the infection in the lungs. A comparison of the median values of each series between 4 and 6 h after infection (i.e., between 2 and 4 h after bacteriophage treatment) showed a decrease in luminescence in

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the animals treated with the bacteriophages of the PAK_Px group (Fig. 2B). For LBL3, luminescence did not begin to decrease until 6 h postinfection. For LUZ19, a decrease in the amount of light emitted was observed between 4 and 8 h after infection, and for PhiKZ, the light emitted from the chest area increased steadily during the first 8 h after infection, as for the PBS group (Fig. 2B). Based on these quantifications and the survival rates obtained, we hypothesized that the amount of light emitted during the first 8 h of infection could be used to anticipate the overall *in vivo* efficacy of a given bacteriophage by defining a predictive index from the ratio of the luminescence values obtained at 2 and 8 h postinfection. The luminescence measured at 2 h reflected the bacterial load at the time of treatment, whereas the largest differences in luminescence between groups were revealed at the 8 h time point. By plotting values of this ratio on a graph, we could discriminate bacteriophage-treated groups into two profiles: the first, with ratio values falling below 3, corresponded to the groups of animals for which bacteriophage treatment resulted in survival rates of at least 75%. The second, for which ratio values remained above 3, corresponded to the groups for which bacteriophage treatment resulted in the survival of no more than 50% of the animals (Fig. 2C). A ratio value of 3 was then chosen as the index to discriminate bacteriophages on the basis of *in vivo* efficacy.

Conflicting results *in vitro* and *in vivo* for bacteriophage PhiKZ. *In vitro* (EOP, lysis kinetics) and *in vivo* efficiencies were highly correlated for all bacteriophages except PhiKZ. Despite having an EOP of 1.2 on the PAK-lumi strain and reasonably good lysis kinetics, survival rates following treatment with PhiKZ were as low as those for PBS when animals were infected at an MOI of 0.1 (Fig. 1B). We investigated the possibility that this bacteriophage was devoid of activity *in vivo* by treating animals with a higher dose (MOI, 20). The survival curves obtained in two independent experiments (Fig. 3B) revealed a dose-dependent effect, as 100% of the animals treated with PhiKZ at an MOI of 20 survived acute infections and displayed a luminescence pattern similar to that obtained for the reference bacteriophage, PAK_P1 (Fig. 3A). In addition, the ratio values calculated from data obtained in these experiments were shown to be consistent with the survival rates obtained (Fig. 3C).

Assessing the reliability of the predictive index. First confirmed by the above-described experiments with PhiKZ, we further assessed the robustness of the index and its applicability to new bacteriophages using CHA_P1, a bacteriophage infecting strain CHA whose genome revealed close similarity to the PAK_P3 genome (these bacteriophages share 90% homology in their proteins). The CHA_P1 bacteriophage, shown to rescue CHA-infected mice, was also able to infect the PAK-lumi strain with an EOP of 0.5 (Fig. 4 and Table 1). The lysis kinetics for CHA_P1 were similar to those for the PAK_Px bacteriophages, although lysis was slightly less rapid (Fig. 5A). These favorable *in vitro* results were not confirmed when CHA_P1 was tested *in vivo*. Surprisingly, luminescence analysis and the calculated ratio of emitted light allowed us to anticipate a low *in vivo* efficacy for CHA_P1 with a value far above the index (Fig. 5B and 3C), which was confirmed by the death of all the treated animals at the end of the experiment (Fig. 5C). We then hypothesized that amplifying this bacteriophage on the PAK strain might increase its efficacy *in vivo*, as previously demonstrated for the *in vitro* adaptation of the PAK_P3 bacteriophage to the CHA strain (32). However, five successive passages on the PAK strain did not significantly increase the EOP on the PAK strain (while the EOP on CHA remained unchanged) or the *in vivo* efficacy of the adapted CHA_P1 bacteriophage against this strain (the survival rate stayed below 25% with treatment at an MOI of 0.1).

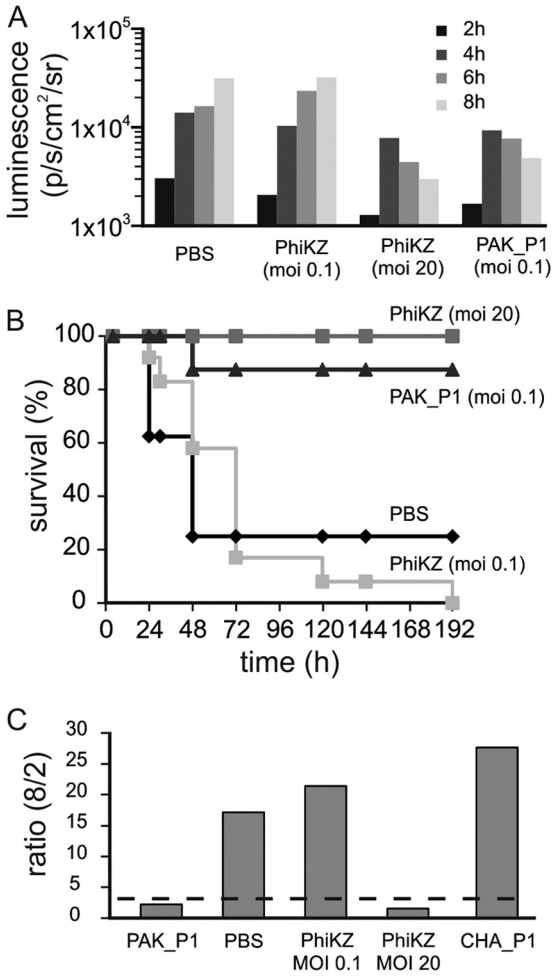

FIG 3 Short-term kinetics and survival curves for PhiKZ-treated mice infected with the PAK-lumi strain. (A, B) Luminescence measurements and survival rates of the groups of mice treated with PhiKZ at the indicated MOIs; (C) ratios of radiance at 8 h postinfection to that at 2 h postinfection in groups treated with PhiKZ and CHA_P1 at the indicated MOIs. For each group, the results shown are from two independent experiments ($n = 8$ to 12).

DISCUSSION

Do all bacteriophages forming plaques on a bacterial lawn qualify as therapeutic candidates? From theoretical studies, several parameters have been proposed to be indicative of the *in vivo* activity of bacteriophages (17, 38), but to our knowledge, only one study has recently tried to tackle this question using an animal model (14). However, in the aforementioned study, bacteriophage and bacterial solutions were simultaneously intramuscularly injected into mice either separately or mixed, leading to the rapid adsorption of bacteriophages to their bacterial host before the infection proceeded. Such a protocol, set for kinetic studies, was not directly relevant to comparison of therapeutic efficacies, as the infection had not started before the treatment was applied. Other studies have also shown in the gut, for example, that it proved difficult to

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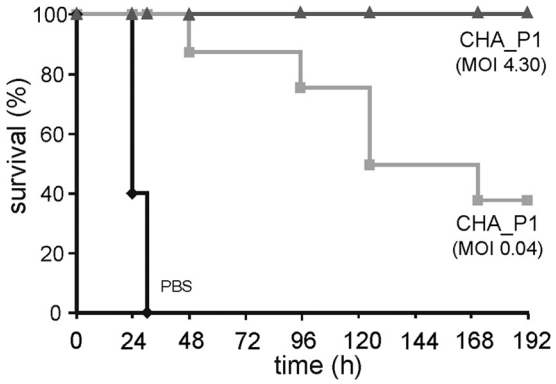

FIG 4 *In vivo* efficacy of the CHA_P1 bacteriophage in CHA-infected animals. Survival curves are for the groups of animals infected with CHA_P1 (4×10^6 CFU) and treated with the CHA_P1 bacteriophage at the indicated MOIs (n = 4 to 8).

predict the *in vivo* efficacy of bacteriophages from *in vitro* observations (24, 37, 39).

Using a single bacterial host, a well-established mouse lung infection model, a real-time imaging system, and a treatment strategy that could be adapted to humans in a context of active therapy (minimal dose and an effect relying on the amplification of the bacteriophage on the site of the infection), we evaluated the *in vitro* and *in vivo* efficacies of a set of nine bacteriophages from various genera.

While exploiting the bioluminescence data, the ratio of light emitted at 2 h to that emitted at 8 h following infection allowed us to discriminate between the most and the least efficient bacteriophages *in vivo*. An index value of 3 was then proposed as a reference to classify these bacteriophages into two groups. We noticed that this index appeared to be reliable, despite the high variability of the ratio values obtained from different rounds of experiments, a characteristic of *in vivo* experimentation (see the results for the PBS groups in both Fig. 2C and 3C). Interestingly, this index allowed us to anticipate the rate of survival as the final outcome of the animal trial, which provides an opportunity to reduce the time and cost of future experiments.

Despite various host factors (naturally present or induced by the infection, such as immune cells) that could jeopardize the success of bacteriophage treatments, we observed a correlation between *in vitro* and *in vivo* efficacy for most of the bacteriophages studied here (with the exception of PhiKZ and CHA_P1). *In vitro* and *in vivo* results were well correlated for the PAK_Px bacteriophages. Among these, variations in efficacy *in vivo* reflected their genetic relatedness (PAK_P3 and PAK_P5 are genetically closely related to each other, as are PAK_P1, PAK_P2, and PAK_P4; Henry et al., unpublished). *In vitro* and *in vivo* results were also well correlated for LBL3 and LUZ19, which were less efficient under both conditions, thus showing that for the majority of bacteriophages considered in this study, *in vitro* data were indicative of *in vivo* efficacy.

Moreover, the optimal efficacy achieved with the bacteriophages isolated on the strain used in the animal model highlights a possible effect of the bacterial host used for isolation on the efficacy of the treatment. This was further supported by the use of CHA_P1, a bacteriophage which is genetically closely related to PAK_P3 and PAK_P5, although it was isolated using the CHA

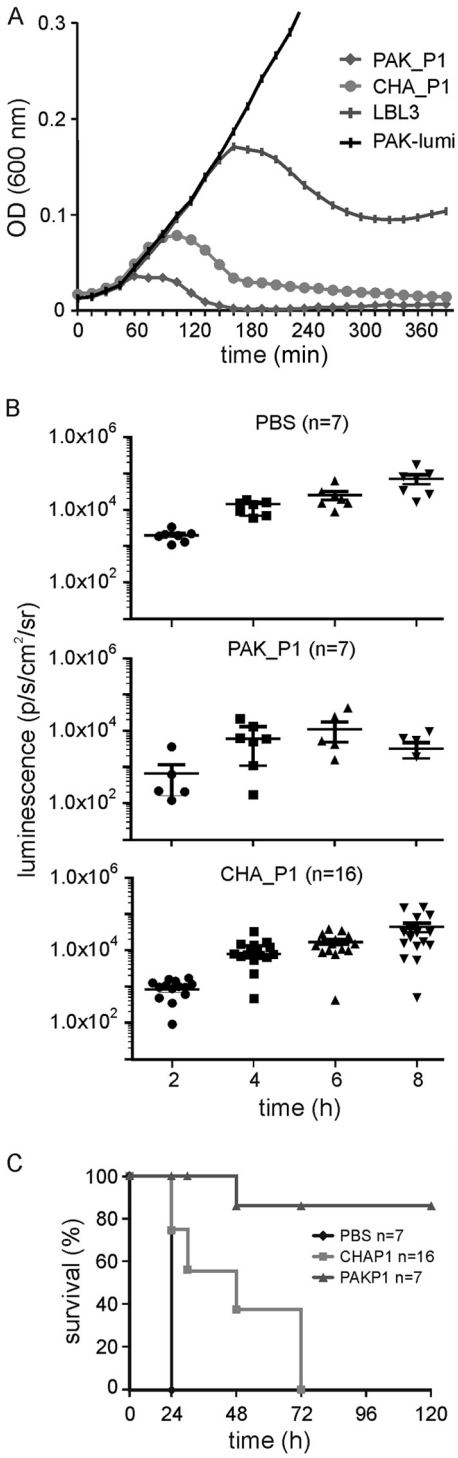

FIG 5 *In vitro* and *in vivo* efficacies of the CHA_P1 bacteriophage on the PAK-lumi strain. (A) Individual lysis kinetics for the indicated bacteriophages at an MOI of 0.001 (values are means of 4 replicates; only one set of data representative of 3 independent experiments is shown) on the PAK-lumi strain; (B) average radiances of the animals used for the evaluation of CHA_P1 treatment at an MOI of 0.1, with medians and interquartile ranges indicated; (C) survival curves of the groups of animals infected with PAK-lumi and treated with the indicated bacteriophages at an MOI of 0.1.

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strain as a host, suggesting that bacteriophages presenting the best chances to be efficient *in vivo* are likely to be those isolated on purpose. Despite this genetic closeness and kinetics of lysis similar to that of PAK_Px, CHA_P1 was unable to cure animals infected with the PAK-lumi strain. In addition, an attempt to adapt this bacteriophage to the PAK strain failed, with neither an increase in EOP nor an improvement in *in vivo* efficacy being achieved. In contrast, CHA_P1 was able to rescue CHA-infected mice, showing that this bacteriophage is nonetheless able to reach and infect bacteria *in vivo*. Similarly, the lack of *in vivo* efficacy of the PhiKZ bacteriophage could not be accounted for by an intrinsic inability to infect bacteria *in vivo*, since increasing the treatment dose allowed maximum efficacy to be reached. These observations warrant further investigation of the factors which are preventing the CHA_P1 bacteriophage from being efficient in PAK-infected animals. These factors could be linked either to the animal host response toward a different bacterial pathogen or to the bacterium itself. For example, the PAK strain could, *in vivo*, repress the expression of a protein required for an optimal infectious cycle of CHA_P1 but not required by the PAK_Px bacteriophages. Our results therefore show that *in vitro* efficacy, although necessary, is not sufficient to ensure *in vivo* efficacy.

To conclude, the results of this study, which for the first time compares the *in vivo* efficacy of various bacteriophages, whether or not they are specifically isolated using the targeted host, illustrate the two complementary strategies currently proposed for use in the development of bacteriophage therapy (40, 41). Depending on the type of infection targeted (chronic or acute) and the patient clinical state, the use of *prêt à porter* (ready-to-use) cocktails aiming to secure minimal efficacy may then be preferred or not over *sur mesure* (personalized) therapy (isolation of bacteriophages using the strain from the patient). The latter is more time consuming, but on the basis of our data suggesting a link between *in vivo* efficacy and the host of isolation, it is favored for optimum efficiency. Our findings may also shed light on possible reasons for the failure of certain bacteriophage therapy trials (experimental or human) when bacteriophages are selected solely on the basis of *in vitro* data. This means that the inability of a bacteriophage active *in vitro* to work in a clinical trial may not imply that the phage therapy treatment cannot work.

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